PATENT

IN UNITED STATES PATENT AND TRADEMARK OFFICE

Patent No.:	7,208,285	Docket No: 17452 (BOT)
Issue Date:	April 24, 2007	Patentee: Lance E. Steward et al.
Title	FRET PROTEASE ASSAYS FOR BOTULINUM SEROTYPE A/E TOXINS	

REQUEST FOR CERTIFICATION OF CORRECTION

Attn: Certificate of Correction Branch
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It is requested that a Certificate of Correction be issued correcting printing errors appearing in the above-identified United States patent. Two copies of the text of the Certificate in the suggested form are enclosed.

Pursuant to 1.20(a), the examiner is authorized to charge the Certificate of Correction fee of $100.00 to the Deposit Account No. 01-0885.

Issuance of the Certificate of Correction would neither expand nor contract the scope of the claims as properly allowed, and re-examination is not required.

The Examiner is authorized to charge any additional fees or credit overpayment to Deposit Account No. 01-0885.

Respectfully submitted,

Date: June 1, 2007

/Dean G. Stathakis/
Attorney Name: Dean G. Stathakis
Reg. No.: 54,465

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UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO : 7,208,285

DATED : April 24, 2007

INVENTOR(S) : Steward et al.

It is certified that errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the face page, in field (56), under "Other Publications", in column 1, lines 1–2, delete "Transfer",Current" and insert - - Transfer", Current - -, therefor.

On the face page, in field (56), under "Other Publications", in column 2, line 2, delete "Release" and insert - - Releases - -, therefor.

On page 2, in field (56), under "Other Publications", in column 1, line 10, delete "Biophyics" and insert - - Biophysics - -, therefor.

On page 2, in field (56), under "Other Publications", in column 1, line 17, delete "Sco." and insert - - Soc. - -, therefor.

On page 2, in field (56), under "Other Publications", in column 2, line 25, delete "C and" and insert - - C, and - -, therefor.

In column 2, line 52, delete "QSY 7®" and insert - - QSY® 7 - -, therefor.

In column 15, line 11, delete "Try" and insert - - Tyr - -, therefor.

In column 18, line 1, delete "5 sec^{-}" and insert - - 5 sec^{-1} - -, therefor.

In column 18, line 2, delete "1000 sec^{-}" and insert - - 1000 sec^{-1} - -, therefor.

In column 18, line 37, delete "cylized" and insert - - cyclized - -, therefor.

In column 20, line 13, after "5,965,699" delete ")".

In columns 21–22, line 4 (Table 2), after "sea urchin" delete "SNAP-23" and insert - - SNAP-25 - -, therefor.

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INVENTOR(S) : Steward et al.

It is certified that errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In columns 21–22, line 11 (Table 2), delete “suceptible” and insert - - susceptible - -, therefor.

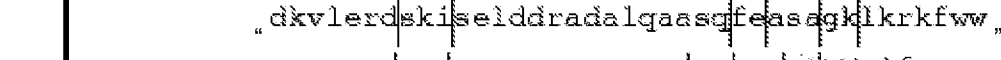
In column 22, line 35, delete “EoNT/A” and insert - - BoNT/A - -, therefor.

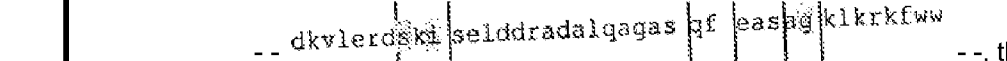
In column 23, line 2, delete “EoNT/A” and insert - - BoNT/A - -, therefor.

In columns 25–26, line 3 (Table 4), below “Species” delete “BoNT/F”.

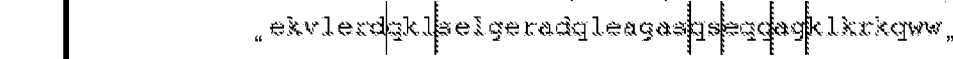
In columns 25–26, line 6 (Table 4), after “TeNT &” delete “BONT/B” and insert - - BoNT/B - -, therefor.

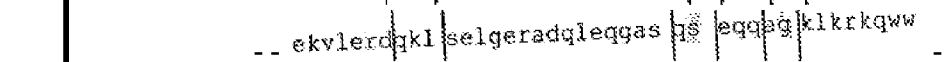
In columns 25–26, line 15 (Table 4), delete

“  ” and insert

- -  - -, therefor.

In columns 25–26, line 17 (Table 4), delete

“  ” and insert

- -  - -, therefor.

In column 30, line 64, delete “BONT A” and insert - - BoNT/A - -, therefor.

In column 31, line 52, after “((SEQ ID NO: 7” delete “;” and insert - -); - -, therefor.

In column 38, line 35, delete “dinitrophneyl” and insert - - dinitrophenyl - -, therefor.

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PATENT NO : 7,208,285

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INVENTOR(S) : Steward et al.

It is certified that errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In column 38, line 40, delete “thiosemicarazide” and insert - - thiosemicarbazide - -, therefor.

In column 38, line 44, delete “napthlene” and insert - - naphthalene - -, therefor.

In column 39, line 52, after “indacene” insert - -) - -.

In column 39, line 56, after “indacene” insert - -) - -.

In column 39, line 57, after “indacene” insert - -) - -.

In column 43, line 49, delete “(Kaschke” and insert - - Kaschke - -, therefor.

In column 45, line 28, delete “(see” and insert - - see - -, therefor.

In column 56, line 8, delete “$_{\lambda Em}$=520” and insert - - λ_{Em}=520 - -, therefor.

In column 113, line 37, in Claim 48, delete “with in” and insert - - within - -, therefor.

In column 114, line 11, in Claim 58, delete “P_3’P_4’” and insert - - P_3’-P_4’ - -, therefor.

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(12) **United States Patent**
Steward et al.

(10) **Patent No.: US 7,208,285 B2**
(45) **Date of Patent: Apr. 24, 2007**

(54) **FRET PROTEASE ASSAYS FOR BOTULINUM SEROTYPE A/E TOXINS**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 346 days.

(21) Appl. No.: **09/942,024**

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US 2003/0143650 A1 Jul. 31, 2003

(51) **Int. Cl.**

C12Q 1/37	(2006.01)
C07H 21/04	(2006.01)
C12P 21/06	(2006.01)
C12N 15/74	(2006.01)
C07N 14/435	(2006.01)

(52) **U.S. Cl.** **435/7.32**; 435/23; 435/69.1; 435/252.3; 435/471; 530/350; 536/23.7

(58) **Field of Classification Search** 435/23, 435/4, 7.1, 7.4, 7.32; 530/326
See application file for complete search history.

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Primary Examiner—Robert A. Zeman
(74) *Attorney, Agent, or Firm*—Dean G. Stathakis; Joel B. German; Martin A. Voet

(57) **ABSTRACT**

The present invention provides clostridial toxin substrates useful in assaying for the protease activity of any clostridial toxin, including botulinum toxins of all serotypes as well as tetanus toxins. A clostridial toxin substrate of the invention contains a donor fluorophore; an acceptor having an absorbance spectrum overlapping the emission spectrum of the donor fluorophore; and a clostridial toxin recognition sequence that includes a cleavage site, where the cleavage site intervenes between the donor fluorophore and the acceptor and where, under the appropriate conditions, resonance energy transfer is exhibited between the donor fluorophore and the acceptor.

88 Claims, 7 Drawing Sheets

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FRET PROTEASE ASSAYS FOR BOTULINUM SEROTYPE A/E TOXINS

FIELD OF THE INVENTION

The present invention relates generally to fluorescence resonance energy transfer and protease assays, for example, assays for protease activity of clostridial toxins such botulinum toxins and tetanus toxins, and more specifically, to intramolecularly quenched substrates and methods for assaying for clostridial toxin protease activity.

BACKGROUND INFORMATION

The neuroparalytic syndrome of tetanus and the rare but potentially fatal disease, botulism, are caused by neurotoxins produced by bacteria of the genus *Clostridium*. These clostridial neurotoxins are highly potent and specific poisons of neural cells, with the human lethal dose of the botulinum toxins on the order of micrograms. Thus, the presence of even minute levels of botulinum toxins in foodstuffs represents a public health hazard that must be avoided through rigorous testing.

However, in spite of their potentially deleterious effects, low controlled doses of botulinum neurotoxins have been successfully used as therapeutics. These toxins have been used in the therapeutic management of a variety of focal and segmental dystonias, of strabismus and other conditions in which a reversible depression of a cholinergic nerve terminal activity is desired. Established therapeutic uses of botulinum neurotoxins in humans include, for example, blepharospasm, hemifacial spasm, laringeal dysphonia, focal hyperhidrosis, hypersalivation, oromandibular dystonia, cervical dystonia, torticollis, strabismus, limbs dystonia, occupational cramps and myokymia (Rossetto et al, *Toxicon* 39:27–41 (2001)). Intramuscular injection of spastic tissue with small quantities of BoNT/A, for example, has been used effectively to treat spasticity due to brain injury, spinal cord injury, stroke, multiple sclerosis and cerebral palsy. Additional possible clinical uses of clostridial neurotoxins currently are being investigated.

Given the potential danger associated with small quantities of botulinum toxins in foodstuffs and the need to prepare accurate pharmaceutical formulations, assays for botulinum neurotoxins presently are employed in both the food and pharmaceutical industry. The food industry requires assays for the botulinum neurotoxins to validate new food packaging methods and to ensure food safety. The growing clinical use of the botulinum toxins necessitates accurate assays for botulinum neurotoxin activity for product formulation as well as quality control. In both industries, a mouse lethality test currently is used to assay for botulinum neurotoxin activity. Unfortunately, this assay suffers from several drawbacks: cost due to the large numbers of laboratory animals required; lack of specificity; and the potential for inaccuracy unless large animal groups are used.

Thus, there is a need for new materials and methods for assaying for clostridial toxin activity. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

The present invention provides clostridial toxin substrates useful in assaying for the protease activity of any clostridial toxin, including botulinum toxins of all serotypes as well as tetanus toxins. A clostridial toxin substrate of the invention contains a donor fluorophore; an acceptor having an absorbance spectrum overlapping the emission spectrum of the donor fluorophore; and a clostridial toxin recognition sequence that includes a cleavage site, where the cleavage site intervenes between the donor fluorophore and the acceptor and where, under the appropriate conditions, resonance energy transfer is exhibited between the donor fluorophore and the acceptor. Such a clostridial toxin substrate can include, for example, a botulinum toxin recognition sequence. In one embodiment, a clostridial toxin substrate of the invention includes a botulinum toxin recognition sequence which is not a botulinum toxin serotype B (BoNT/B) recognition sequence.

The invention also provides a botulinum serotype A/E (BoNT/A/E) substrate containing (a) a donor fluorophore; (b) an acceptor having an absorbance spectrum overlapping the emission spectrum of the donor fluorophore; and (c) a BoNT A or BoNT/E recognition sequence containing a cleavage site, where the cleavage site intervenes between the donor fluorophore and the acceptor and where, under the appropriate conditions, resonance energy transfer is exhibited between the donor fluorophore and the acceptor. Such a botulinum serotype A/E substrate also can be susceptible to cleavage by both the BoNT/A and BoNT/E toxins.

The invention further provides, for example, a botulinum toxin serotype A (BoNT/A) substrate containing a donor fluorophore; an acceptor having an absorbance spectrum overlapping the emission spectrum of the donor fluorophore; and a BoNT/A recognition sequence that includes a cleavage site, where the cleavage site intervenes between the donor fluorophore and the acceptor and where, under the appropriate conditions, resonance energy transfer is exhibited between the donor fluorophore and the acceptor. A BoNT/A substrate of the invention can include, for example, at least six consecutive residues of synaptosome-associated protein of 25 kDa (SNAP-25), where the six consecutive residues include Gln-Arg, or a peptidomimetic thereof. In these and other amino acid sequences provided herein, it is understood that the sequence is written in the direction from N-terminus to C-terminus. A BoNT/A substrate of the invention also can have, for example, at least six consecutive residues of human SNAP-25, where the six consecutive residues include Gln_{197}-Arg_{198}, or a peptidomimetic thereof. In one embodiment, a BoNT/A substrate of the invention includes the amino acid sequence Glu-Ala-Asn-Gln-Arg-Ala-Thr-Lys (SEQ ID NO: 1), or a peptidomimetic thereof. In another embodiment, a BoNT/A substrate of the invention includes residues 187 to 203 of human SNAP-25 (SEQ ID NO: 2), or a peptidomimetic thereof. A variety of donor fluorophores and acceptors are useful in a BoNT/A substrate of the invention, including but not limited to, fluorescein-tetramethylrhodamine; DABCYL-EDANS; and Alexa Fluor® 488 QSY 7®.

Further provided by the invention is a botulinum toxin serotype B (BoNT/B) substrate containing a donor fluorophore; an acceptor having an absorbance spectrum overlapping the emission spectrum of the donor fluorophore; and a BoNT/B recognition sequence that includes a cleavage site, where the cleavage site intervenes between the donor fluorophore and the acceptor and where, under the appropriate conditions, resonance energy transfer is exhibited between the donor fluorophore and the acceptor. A BoNT/B substrate of the invention can contain, for example, at least six consecutive residues of vesicle-associated membrane protein (VAMP), where the six consecutive residues include Gln-Phe, or a peptidomimetic thereof. For example, a BoNT/B substrate of the invention can contain at least six consecutive residues of human VAMP-2, the six consecutive

TABLE 1

Bond cleaved in human VAMP-2, SNAP-25 or syntaxin

Toxin	Target	P_4-P_3-P_2-P_1 -- P_1'-P_2'-P_3'-P_4'
BoNT/A	SNAP-25	Glu-Ala-Asn-Gln-Arg*-Ala-Thr-Lys SEQ ID NO: 1
BoNT/B	VAMP-2	Gly-Ala-Ser-Gln-Phe*-Glu-Thr-Ser SEQ ID NO: 3
BoNT/C1	syntaxin	Asp-Thr-Lys-Lys-Ala*-Val-Lys-Try SEQ ID NO: 5
BoNT/D	VAMP-2	Arg-Asp-Gln-Lys-Leu*-Ser-Glu-Leu SEQ ID NO: 6
BoNT/E	SNAP-25	Gln-Ile-Asp-Arg-Ile*-Met-Glu-Lys SEQ ID NO: 8
BoNT/F	VAMP-2	Glu-Arg-Asp-Gln-Lys*-Leu-Ser-Glu SEQ ID NO: 9
BoNT/G	VAMP-2	Glu-Thr-Ser-Ala-Ala*-Lys-Leu-Lys SEQ ID NO: 10
TeNT	VAMP-2	Gly-Ala-Ser-Gln-Phe*-Glu-Thr-Ser SEQ ID NO: 11

*Scissile bond shown in bold

SNAP-25, VAMP and syntaxin share a short motif located within regions predicted to adopt an α-helical conformation (see FIG. **4**). This motif is present in SNAP-25, VAMP and syntaxin isoforms expressed in animals sensitive to the neurotoxins. In contrast, *Drosophila* and yeast homologs that are resistant to these neurotoxins and syntaxin isoforms not involved in exocytosis contain sequence variations in the α-helical motif regions of these VAMP and syntaxin proteins.

Multiple repetitions of the α-helical motif are present in proteins sensitive to cleavage by clostridial toxins: four copies are naturally present in SNAP-25; two copies are naturally present in VAMP; and two copies are naturally present in syntaxin (see FIG. **4**A). Furthermore, peptides corresponding to the specific sequence of the α-helical motifs can inhibit neurotoxin activity in vitro and in vivo, and such peptides can cross-inhibit different neurotoxins. In addition, antibodies raised against such peptides can cross-react among the three target proteins, indicating that this α-helical motif is exposed on the cell surface and adopts a similar configuration in each of the three target proteins. Consistent with these findings, SNAP-25-specific, VAMP-specific and syntaxin-specific neurotoxins cross-inhibit each other by competing for the same binding site, although they do not cleave targets non-specifically. These results indicate that a clostridial toxin recognition sequence can include, if desired, at least one α-helical motif. It is recognized that an α-helical motif is not absolutely required for cleavage by a clostridial toxin as evidenced by 16-mer and 17-mer substrates for BoNT/A, as discussed further below.

Although multiple α-helical motifs are found in SNAP-25, VAMP and syntaxin, in one embodiment the invention provides a clostridial toxin substrate in which the clostridial toxin recognition sequence includes a single α-helical motif. In another embodiment, the invention provides a clostridial toxin substrate in which the clostridial toxin recognition sequence includes two or more α-helical motifs. A BoNT/A or BoNT/E recognition sequence can include, for example, the S4 α-helical motif, alone or combined with one or more additional α-helical motifs; BoNT/B, BoNT/G or TeNT recognition sequence can include, for example, the V2 α-helical motif, alone or combined with one or more additional α-helical motifs; a BoNT/C1 recognition sequence can include, for example, the S4 α-helical motif, alone or combined with one or more additional α-helical motifs, or X2 α-helical motif, alone or combined with one or more additional α-helical motifs; and a BoNT/D or BoNT/F recognition sequence can include, for example, the V1 α-helical motif, alone or combined with one or more additional α-helical motifs (see FIG. **4**A).

A clostridial toxin substrate of the invention can contain one or multiple clostridial toxin cleavage sites for the same or different clostridial toxin. In one embodiment, a clostridial toxin substrate of the invention contains a single cleavage site. In another embodiment, a clostridial toxin substrate of the invention has multiple cleavage sites for the same clostridial toxin. These cleavage sites can be accompanied by the same or different clostridial toxin recognition sequences. In a further embodiment, a clostridial toxin substrate of the invention has multiple cleavage sites for the same clostridial toxin that intervene between the same donor fluorophore and acceptor. A clostridial toxin substrate of the invention can contain, for example, two or more, three or more, five or more, or ten or more cleavage sites for the same clostridial toxin intervening between the same or different donor fluorophore-acceptor pairs. A clostridial substrate of the invention also can have, for example, two, three, four, five, six, seven, eight, nine or ten cleavage sites for the same clostridial toxin intervening between the same or different donor fluorophore-acceptor pairs.

A clostridial toxin substrate of the invention containing multiple cleavage sites can contain cleavage sites and recognition sequences for different clostridial toxins. In one embodiment, a clostridial toxin substrate of the invention includes multiple cleavage sites for different clostridial toxins all intervening between the same donor fluorophore-acceptor pair. A clostridial toxin substrate of the invention can contain, for example, two or more, three or more, five or more, or ten or more cleavage sites for different clostridial toxins all intervening between the same donor fluorophore-acceptor pair. A clostridial toxin substrate of the invention also can contain, for example, two or more, three or more, five or more, or ten or more cleavage sites for different clostridial toxins intervening between at least two donor fluorophore-acceptor pairs. In particular embodiments, a clostridial substrate of the invention also has two, three, four, five, six, seven, eight, nine or ten cleavage sites for different clostridial toxins, where the cleavage sites intervene between the same or different donor fluorophore-acceptor pairs. A clostridial toxin substrate of the invention having multiple cleavage sites can have, for example, any combination of two, three, four, five, six, seven or eight cleavage sites for any combination of the following clostridial toxins: BoNT/A, BoNT/B, BoNT/C1, BoNT/D, BoNT/E, BoNT/F, BoNT/G and TeNT.

It is understood that a clostridial toxin substrate of the invention can be cleaved at a reduced or enhanced rate relative to SNAP-25, VAMP or syntaxin or relative to a similar peptide or peptidomimetic that does not contain extrinsic fluorophores. A clostridial toxin substrate of the invention such as a BoNT/A, BoNT/B, BoNT/C1 , BoNT/D, BoNT/E, BoNT/F, BoNT/G or TeNT substrate, can be cleaved, for example, with an initial hydrolysis rate that is at least 5% of the initial hydrolysis rate, under otherwise identical conditions, of human SNAP-25, VAMP or syn-

taxin, where the clostridial toxin substrate and SNAP-25, VAMP or syntaxin each is present at a concentration of 1.0 mM.

Thus, a BoNT/A, BoNT/C1 or BoNT/E substrate of the invention can be cleaved, for example, with an initial hydrolysis rate that is at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, 250%, or 300% of the initial hydrolysis rate, under otherwise identical conditions, of human SNAP-25 by BoNT/A, BoNT/C1 or BoNT/E, respectively, where the substrate of the invention and human SNAP-25 each is present at a concentration of 1.0 mM. In other embodiments, a BoNT/A, BoNT/C1 or BoNT/E substrate of the invention is with an initial hydrolysis rate that is at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, 250%, or 300% of the initial hydrolysis rate, under otherwise identical conditions, of human SNAP-25 by BoNT/A, BoNT/C1 or BoNT/E, respectively, where the substrate of the invention and human SNAP-25 each is present at a concentration of 50 mM.

Similarly, a BoNT/B, BoNT/D, BoNT/F or BoNT/G substrate of the invention can be cleaved, for example, with an initial hydrolysis rate that is at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, 250%, or 300% of the initial hydrolysis rate, under otherwise identical conditions, of human VAMP-2 by BoNT/B, BoNT/D, BoNT/F or BoNT/G, respectively, where substrate of the invention and human VAMP-2 each is present at a concentration of 1.0 mM. In other embodiments, a BoNT/B, BoNT/D, BoNT/F or BoNT/G substrate of the invention is cleaved with an initial hydrolysis rate that is at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, 250%, or 300% of the initial hydrolysis rate, under otherwise identical conditions, of human VAMP-2 by BoNT/B, BoNT/D, BoNT/F or BoNT/G, respectively, where substrate of the invention and human VAMP-2 each is present at a concentration of 50 mM.

The invention also provides a BoNT/C1 substrate of the invention that is cleaved with an initial hydrolysis rate that is at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, 250%, or 300% of the initial hydrolysis rate, under otherwise identical conditions, of human syntaxin by BoNT/C1, where the BoNT/C1 substrate and human syntaxin each is present at a concentration of 1.0 mM. In other embodiments, the invention provides a BoNT/C1 substrate that is cleaved with an initial hydrolysis rate that is at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, 250%, or 300% of the initial hydrolysis rate, under otherwise identical conditions, of human syntaxin by BoNT/C1, where the BoNT/C1 substrate and human syntaxin each is present at a concentration of 50 mM.

The "turnover number," or k_{cat}, is the rate of breakdown of a toxin-substrate complex. A clostridial toxin substrate of the invention can be cleaved with a k_{cat} that is reduced or enhanced as compared to the k_{cat} of human SNAP-25, human VAMP-2 or human syntaxin target proteins when cleaved by the same clostridial toxin under the same conditions. A clostridial toxin substrate of the invention such as a BoNT/A, BoNT/B, BoNT/C1, BoNT/D, BoNT/E, BoNT/F, BoNT/G or TeNT substrate, can be cleaved, for example, with a k_{cat} of about 0.001 to about 4000 sec^{-1}. In one embodiment, a clostridial toxin substrate of the invention such as a BoNT/A, BoNT/B, BoNT/C1, BoNT/D, BoNT/E, BoNT/F, BoNT/G or TeNT substrate is cleaved with a k_{cat} of about 1 to about 4000 sec^{-1}. In other embodiments, a BoNT/A, BoNT/B, BoNT/C1, BoNT/D, BoNT/E, BoNT/F, BoNT/G or TeNT substrate of the invention has a k_{cat} of less than 5 sec^{-1}, 10 sec^{-1}, 25 sec^{-1}, 50 sec^{-1}, 100 sec^{-1}, 250 sec^{-1}, 500 sec^{-1}, or 1000 sec^{-1}. A clostridial toxin substrate of the invention such as a BoNT/A, BoNT/B, BoNT/C1, BoNT/D, BoNT/E, BoNT/F, BoNT/G or TeNT substrate also can have, for example, a k_{cat} in the range of 1 to 1000 sec^{-1}; 1 to 500 sec^{-1}; 1 to 250 sec^{-1}; 1 to 100 sec^{-1}; 1 to 50 sec^{-1}; 10 to 1000 sec^{-1}; 10 to 500 sec^{-1}; 10 to 250 sec^{-1}; 10 to 100 sec^{-1}; 10 to 50 sec^{-1}; 25 to 1000 sec^{-1}; 25 to 500 sec^{-1}; 25 to 250 sec^{-1}; 25 to 100 sec^{-1}; 25 to 50 sec^{-1}; 50 to 1000 sec^{-1}; 50 to 500 sec^{-1}; 50 to 250 sec^{-1}; 50 to 100 sec^{-1}; 100 to 1000 sec^{-1}; 100 to 500 sec^{-1}; or 100 to 250 sec^{-1}. One skilled in the art understands the turnover number, k_{cat}, is assayed under standard kinetic conditions in which there is an excess of substrate.

In particular embodiments, a clostridial toxin substrate of the invention is a peptide or peptidomimetic. As used herein, the term "peptidomimetic" is used broadly to mean a peptide-like molecule that is cleaved by the same clostridial toxin as the peptide substrate upon which it is structurally based. Such peptidomimetics include chemically modified peptides, peptide-like molecules containing non-naturally occurring amino acids, and peptoids, which are peptide-like molecules resulting from oligomeric assembly of N-substituted glycines, and are cleaved by the same clostridial toxin as the peptide substrate upon which the peptidomimetic is derived (see, for example, Goodman and Ro, *Peptidomimetics for Drug Design*, in "Burger's Medicinal Chemistry and Drug Discovery" Vol. 1 (ed. M. E. Wolff; John Wiley & Sons 1995), pages 803–861).

A variety of peptidomimetics are known in the art including, for example, peptide-like molecules which contain a constrained amino acid, a non-peptide component that mimics peptide secondary structure, or an amide bond isostere. A peptidomimetic that contains a constrained, non-naturally occurring amino acid can include, for example, an α-methylated amino acid; an α,α-dialkylglycine or α-aminocycloalkane carboxylic acid; an N^{α}-C^{α} cylized amino acid; an N^{α}-methylated amino acid; a β- or γ-amino cycloalkane carboxylic acid; an α,β-unsaturated amino acid; a β,β-dimethyl or β-methyl amino acid; a β-substituted-2,3-methano amino acid; an N-C^{δ} or C^{α}-C^{δ} cyclized amino acid; or a substituted proline or another amino acid mimetic. In addition, a peptidomimetic which mimics peptide secondary structure can contain, for example, a nonpeptidic β-turn mimic; γ-turn mimic; mimic of β-sheet structure; or mimic of helical structure, each of which is well known in the art. A peptidomimetic also can be a peptide-like molecule which contains, for example, an amide bond isostere such as a retro-inverso modification; reduced amide bond; methylenethioether or methylenesulfoxide bond; methylene ether bond; ethylene bond; thioamide bond; trans-olefin or fluoroolefin bond; 1,5-disubstituted tetrazole ring; ketomethylene or fluoroketomethylene bond or another amide isostere. One skilled in the art understands that these and other peptidomimetics are encompassed within the meaning of the term "peptidomimetic" as used herein.

The invention provides, for example, a botulinum toxin serotype A (BoNT/A) substrate containing a donor fluorophore; an acceptor having an absorbance spectrum overlapping the emission spectrum of the donor fluorophore; and a BoNT/A recognition sequence that includes a cleavage site, where the cleavage site intervenes between the donor fluorophore and the acceptor and where, under the appropriate conditions, resonance energy transfer is exhibited between the donor fluorophore and the acceptor. A BoNT/A substrate of the invention can include, for example, at least six consecutive residues of SNAP-25, where the six consecutive

residues include Gln-Arg, or a peptidomimetic thereof. Such a BoNT/A substrate also can have, for example, at least six consecutive residues of human SNAP-25, where the six consecutive residues include Gln_{197}-Arg_{198}, or a peptidomimetic thereof. In one embodiment, a BoNT/A substrate of the invention includes the amino acid sequence Glu-Ala-Asn-Gln-Arg-Ala-Thr-Lys (SEQ ID NO: 1), or a peptidomimetic thereof. In another embodiment, a BoNT/A substrate of the invention includes residues 187 to 203 of human SNAP-25 (SEQ ID NO: 2), or a peptidomimetic thereof. A variety of donor fluorophores and acceptors are useful in a BoNT/A substrate of the invention, including but not limited to, fluorescein-tetramethylrhodamine, DABCYL-EDANS, and Alexa Fluor® 488-QSY® 7. Additional donor fluorophores and acceptors useful in a BoNT/A substrate of the invention are described further herein below.

As used herein, the term "botulinum toxin serotype A recognition sequence" is synonymous with "BoNT/A recognition sequence" and means a scissile bond together with adjacent or non-adjacent recognition elements sufficient for detectable proteolysis at the scissile bond by a BoNT/A under conditions suitable for clostridial toxin protease activity. A scissile bond cleaved by BoNT/A can be, for example, Gln-Arg.

A variety of BoNT/A recognition sequences are well known in the art. A BoNT/A recognition sequence can have, for example, residues 134 to 206 or residues 137 to 206 of human SNAP-25 (Ekong et al., supra, 1997; U.S. Pat. No. 5,962,637). A BoNT/A recognition sequence also can include, without limitation, the sequence Thr-Arg-Ile-Asp-Glu-Ala-Asn-Gln-Arg-Ala-Thr-Lys-Met (SEQ ID NO: 27), or a peptidomimetic thereof, which corresponds to residues 190 to 202 of human SNAP-25; Ser-Asn-Lys-Thr-Arg-Ile-Asp-Glu-Ala-Asn-Gln-Arg-Ala-Thr-Lys (SEQ ID NO: 28), or a peptidomimetic thereof, which corresponds to residues 187 to 201 of human SNAP-25; Ser-Asn-Lys-Thr-Arg-Ile-Asp-Glu-Ala-Asn-Gln-Arg-Ala-Thr-Lys-Met (SEQ ID NO: 29), or a peptidomimetic thereof, which corresponds to residues 187 to 202 of human SNAP-25; Ser-Asn-Lys-Thr-Arg-Ile-Asp-Glu-Ala-Asn-Gln-Arg-Ala-Thr-Lys-Met-Leu (SEQ ID NO: 30), or a peptidomimetic thereof, which corresponds to residues 187 to 203 of human SNAP-25; Asp-Ser-Asn-Lys-Thr-Arg-Ile-Asp-Glu-Ala-Asn-Gln-Arg-Ala-Thr-Lys-Met (SEQ ID NO: 31), or a peptidomimetic thereof, which corresponds to residues 186 to 202 of human SNAP-25; or Asp-Ser-Asn-Lys-Thr-Arg-Ile-Asp-Glu-Ala-Asn-Gln-Arg-Ala-Thr-Lys-Met-Leu (SEQ ID NO: 32), or a peptidomimetic thereof, which corresponds to residues 186 to 203 of human SNAP-25. See, for example, Schmidt and Bostian, *J. Protein Chem.* 14:703–708 (1995); Schmidt and Bostian, supra, 1997; Schmidt et al., *FEBS Letters* 435: 61–64 (1998); and Schmidt and Bostian, U.S. Pat. No. 5,965,698). If desired, a similar BoNT/A recognition sequence can be prepared from a corresponding (homologous) segment of another BoNT/A-sensitive SNAP-25 isoform or homolog such as, for example, murine, rat, goldfish or zebrafish SNAP-25 or can be any of the peptides disclosed herein or described in the art, for example, in U.S. Pat. No. 5,965,699.

A BoNT/A recognition sequence can correspond to a segment of a protein that is sensitive to cleavage by botulinum toxin serotype A, or can be substantially similar to a segment of a BoNT/A-sensitive protein. As illustrated in Table 2, a variety of naturally occurring proteins sensitive to cleavage by BoNT/A are known in the art and include, for example, human, mouse and rat SNAP-25; and goldfish SNAP-25A and SNAP-25B. Thus, a BoNT/A recognition sequence useful in a BoNT/A substrate of the invention can correspond, for example, to a segment of human SNAP-25, mouse SNAP-25, rat SNAP-25, goldfish SNAP-25A or 25B, or another naturally occurring protein sensitive to cleavage by BoNT/A. Furthermore, comparison of native SNAP-25 amino acid sequences cleaved by BoNT/A reveals that such sequences are not absolutely conserved (see Table 2 and FIG. **5**), indicating that a variety of amino acid substitutions and modifications relative to a naturally occurring BoNT/A-sensitive SNAP-25 sequence can be tolerated in a BoNT/A substrate of the invention.

TABLE 2

Cleavage of SNAP-25 and related proteins[a,b,c,d]

Species	Isoform	Cleavage Sites	SEQ ID NO:	Resistance to Cleavage by
		BoNT/E ↓ BoNT/A ↓ ↓ BoNT/C		
human mouse rat	SNAP-25	174 qnrqidrimekadsnktrideanqratkmlgsg 206		none[a]
human	SNAP-23	180 qnqqiqkitdkadtnrdridianarakklids end		all[b]
mouse	SNAP-23	179 qnqqiqkitekadtnknridiantrakklids end		BoNT/A & C
chicken	SNAP-25	174 qnrqidrimeklipikpglmkptsvqqrcsavvk end		BoNT/A & C
goldfish	SNAP-25 A	171 qnrqidrimdmadsnktrideanqratkmlgsg end		none
	SNAP-25 B	172 qnrqidrimekadsnktrideanqratkmlgsg end		none

TABLE 2-continued

Cleavage of SNAP-25 and related proteins[a,b,c,d]

Species	Isoform	Cleavage Sites	SEQ ID NO:	Resistance to Cleavage by
Torpedo	SNAP-25	180 qnaqvdrivvkgdmnkarideankhatkml end		BoNT/E[c] & A[d]
sea urchin	SNAP-23	180 qnsqvgritskaesnegrinsadkraknilrnk end		(?)[e]
C-elegans	SNAP-25	203 qnrqldrihdkqsnevrvesankraknlitk end		BoNT/A & C
Drosophila	SNAP-25	182 qnrqidrinrkgesneariavanqrahqllk end		BoNT/E & A[e]
leech	SNAP-25	181 qnrqvdrinnkmtsnqlrisdankraskllke end		BoNT/A[e]

[a]= In vitro cleavage of SNAP-25 requires 1000-fold higher BoNT/C concentration than BoNT/A or /E.

[b]= Substitution of p182r, or k185dd (boxes) induces susceptibility toward BoNT/E.

[c]= Resistance to BoNT/A possibly due to d189 or e189 substitution by v189, see box.

[d]= Note that Torpedo is suceptible to BoNT/A.

[e]= Note the presence of several non-conservative mutations around putative cleavage sites.

A clostridial toxin substrate of the invention, such as a BoNT/A substrate, can have one or multiple modifications as compared to a naturally occurring sequence that is cleaved by the corresponding clostridial toxin. For example, as compared to a 17-mer corresponding to residues 187 to 203 of human SNAP-25, substitution of Asp193 with Asn in the BoNT/A substrate resulted in a relative rate of proteolysis of 0.23; substitution of Glu194 with Gln resulted in a relative rate of 2.08; substitution of Ala195 with 2-aminobutyric acid resulted in a relative rate of 0.38; and substitution of Gln197 with Asn, 2-aminobutyric acid or Ala resulted in a relative rate of 0.66, 0.25, or 0.19, respectively (see Table 3). Furthermore, substitution of Ala199 with 2-aminobutyric acid resulted in a relative rate of 0.79; substitution of Thr200 with Ser or 2-aminobutyric acid resulted in a relative rate of 0.26 or 1.20, respectively; substitution of Lys201 with Ala resulted in a relative rate of 0.12; and substitution of Met202 with Ala or norleucine resulted in a relative rate of 0.38 or 1.20, respectively. See Schmidt and Bostian, supra, 1997. These results indicate that a variety of residues can be substituted in a clostridial toxin substrate of the invention as compared to a naturally occurring toxin-sensitive sequence. In the case of BoNT/A, these results indicate that residues including but not limited to Glu194, Ala195, Gln197, Ala199, Thr200 and Met202, Leu203, Gly204, Ser205, and Gly206, as well as residues more distal from the Gln-Arg scissile bond can be substituted or can be conjugated to a donor fluorophore or acceptor to produce a BoNT/A substrate of the invention. Such a BoNT/A substrate is detectably proteolyzed at the scissile bond by BoNT/A under conditions suitable for clostridial toxin protease activity. Thus, a BoNT/A substrate of the invention can include, if desired, one or several amino acid substitutions, additions or deletions relative to a naturally occurring SNAP-25 sequence.

TABLE 3

Kinetic parameters of EoNT/A synthetic peptide substrates

Peptide	Sequence[a]	SEQ ID NO:	Relative Rate[b]
[1–15]	SNKTRIDEANQRATK	28	0.03
[1–16]	SNKTRTDEANQRATKM	29	1.17
[1–17]	SNKTRIDEANQRATKML	30	1.00
M16A	SNKTRIDEANQRATK**A**L	44	0.38
M16X	SNKTRIDEANQRATK**X**L	45	1.20
K15A	SNKTRIDEANQRAT**A**ML	46	0.12
T14S	SNKTRIDEANQRA**S**KML	47	0.26
T14B	SNKTRIDEANQRA**B**KML	48	1.20
A13B	SNKTRIDEANQR**B**TKML	49	0.79
Q11A	SNKTRIDEAN**A**RATKML	50	0.19
Q11B	SNKTRIDEAN**B**RATKML	51	0.25
Q11N	SNKTRIDEANN**N**RATKML	52	0.66
N10A	SNKTRIDEA**A**QRATKML	53	0.06
A9B	SNKTRIDE**B**NQRATKML	54	0.38

TABLE 3-continued

Kinetic parameters of EoNT/A synthetic peptide substrates

Peptide	Sequence[a]	SEQ ID NO:	Relative Rate[b]
E8Q	SNKTRIDQANQRATKML	55	2.08
D7N	SNKTRINEANQRATKML	56	0.23

[a]Nonstandard amino acid abbreviations are: B, 2-aminobutyric acid; X, 2-aminohexanoic acid (norleucine)
[b]Initial hydrolysis rates relative to peptide [1–17]. Peptide concentrations were 1.0 mM.

In standard nomenclature, the sequence surrounding clostridial toxin cleavage sites is denoted P_5-P_4-P_3-P_2-P_1-P_1'-P_2'-P_3'-P_4'-P_5', with P_1-P_1' the scissile bond. In one embodiment, the invention provides a BoNT/A substrate or other clostridial toxin substrate in which the residue at position P_1, P_2, P_3, P_4, P_5, or $P_{>5}$ is substituted with an amino acid conjugated to a donor fluorophore or acceptor, and in which the residue at position P_1', P_2', P_3', P_4', P_5' or $P_{>5}$' is substituted with an amino acid conjugated to a donor fluorophore or acceptor. In another embodiment, the invention provides a BoNT/A substrate or other clostridial toxin substrate in which the residue at position P_1, P_3, P_4 or $P_{>5}$ is substituted with an amino acid conjugated to a donor fluorophore or acceptor, and in which the residue at position P_2', P_3', P_5' or $P_{>5}$' is substituted with an amino acid conjugated to a donor fluorophore or acceptor. It is further understood that the amino acid side chain of the residue conjugated to a donor fluorophore or acceptor can be otherwise identical to the residue present in the corresponding position of the naturally occurring target protein, or can contain, for example, a different side chain. Further provided by the invention is a BoNT/A substrate or other clostridial toxin substrate in which the residue at P_3, P_4 or $P_{>5}$ is substituted with an amino acid conjugated to a donor fluorophore or acceptor, and in which the residue at position P_2', P_3', P_5' or $P_{>5}$' is substituted with an amino acid conjugated to a donor fluorophore or acceptor. Again, the amino acid side chain of the residue conjugated to a donor fluorophore or acceptor can be otherwise identical to the residue present in the corresponding position of the naturally occurring target protein, or can contain, for example, a different side chain.

A BoNT/A substrate of the invention also can include, if desired, a carboxy-terminal amide. Thus, a BoNT/A substrate of the invention can be, for example, a peptide having at most twenty, thirty, forty or fifty residues and containing a carboxy-terminal amide.

Further provided by the invention is a botulinum toxin serotype B (BoNT/B) substrate containing a donor fluorophore; an acceptor having an absorbance spectrum overlapping the emission spectrum of the donor fluorophore; and a BoNT/B recognition sequence that includes a cleavage site, where the cleavage site intervenes between the donor fluorophore and the acceptor and where, under the appropriate conditions, resonance energy transfer is exhibited between the donor fluorophore and the acceptor. A BoNT/B substrate of the invention can contain, for example, at least six consecutive residues of VAMP, where the six consecutive residues include Gln-Phe, or a peptidomimetic thereof. For example, a BoNT/B substrate of the invention can contain at least six consecutive residues of human VAMP-2, the six consecutive residues including Gln_{76}-Phe_{77}, or a peptidomimetic thereof. In one embodiment, a BoNT/B substrate includes the amino acid sequence Gly-Ala-Ser-Gln-Phe-Glu-Thr-Ser (SEQ ID NO: 3), or a peptidomimetic thereof. In other embodiments, a BoNT/B substrate includes residues 55 to 94 of human VAMP-2 (SEQ ID NO: 4); residues 60 to 94 of human VAMP-2 (SEQ ID NO: 4); or residues 60 to 88 of human VAMP-2 (SEQ ID NO: 4), or a peptidomimetic of one of these sequences. It is understood that a variety of donor fluorophores and acceptors are useful in a BoNT/B substrate of the invention; such donor fluorophore-acceptor combinations include, but are not limited to, fluorescein-tetramethylrhodamine; DABCYL-EDANS; and Alexa Fluor® 488-QSY® 7. A variety of additional donor fluorophores and acceptors useful in a BoNT/B substrate of the invention are known in the art and described further below.

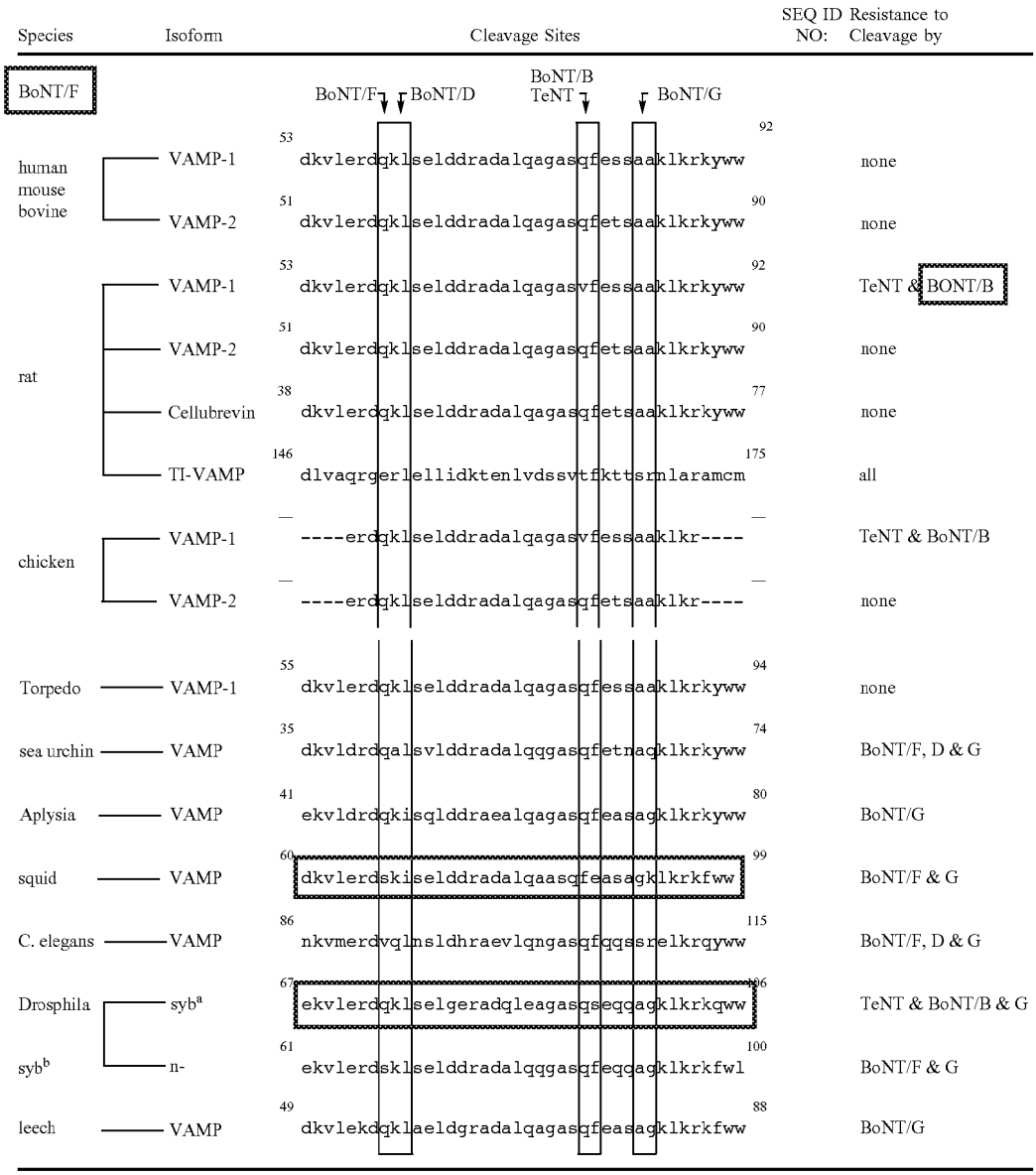
As used herein, the term "botulinum toxin serotype B recognition sequence" is synonymous with "BoNT/B recognition sequence" and means a scissile bond together with adjacent or non-adjacent recognition elements sufficient for detectable proteolysis at the scissile bond by a BoNT/B under appropriate conditions. A scissile bond cleaved by BoNT/B can be, for example, Gln-Phe.

A variety of BoNT/B recognition sequences are well known in the art or can be defined by routine methods. Such a BoNT/B recognition sequence can include, for example, a sequence corresponding to some or all of the hydrophilic core of a VAMP protein such as human VAMP-1 or human VAMP-2. A BoNT/B recognition sequence can include, without limitation, residues 33 to 94, residues 45 to 94, residues 55 to 94, residues 60 to 94, residues 65 to 94, residues 60 to 88 or residues 65 to 88 of human VAMP-2 (SEQ ID NO: 4), or residues 60 to 94 of human VAMP-1 (SEQ ID NO: 96) (see, for example, Shone et al., *Eur. J. Biochem.* 217: 965–971 (1993) and U.S. Pat. No. 5,962,637). If desired, a similar BoNT/B recognition sequence can be prepared from a corresponding (homologous) segment of another BoNT/B-sensitive VAMP isoform or homolog such as human VAMP-1 or rat or chicken VAMP-2.

Thus, it is understood that a BoNT/B recognition sequence can correspond to a segment of a protein that is sensitive to cleavage by botulinum toxin serotype B, or can be substantially similar to such a segment of a BoNT/B-sensitive protein. As shown in Table 4, a variety of naturally occurring proteins sensitive to cleavage by BoNT/B are known in the art and include, for example, human, mouse and bovine VAMP-1 and VAMP-2; rat VAMP-2; rat cellubrevin; chicken VAMP-2; Torpedo VAMP-1; sea urchin VAMP; Aplysia VAMP; squid VAMP; *C. elegans* VAMP; *Drosophila* n-syb; and leech VAMP. Thus, a BoNT/B recognition sequence useful in a BoNT/B substrate of the invention can correspond, for example, to a segment of human VAMP-1 or VAMP-2, mouse VAMP-1 or VAMP-2, bovine VAMP-1 or VAMP-2, rat VAMP-2, rat cellubrevin, chicken VAMP-2, Torpedo VAMP-1, sea urchin VAMP, Aplysia VAMP, squid VAMP, *C. elegans* VAMP, *Drosophila* n-syb, leech VAMP, or another naturally occurring protein sensitive to cleavage by BoNT/B. Furthermore, as shown in Table 4, comparison of native VAMP amino acid sequences cleaved by BoNT/B reveals that such sequences are not absolutely conserved (see, also, FIG. **6**), indicating that a variety of amino acid substitutions and modifications relative to a naturally occurring VAMP sequence can be tolerated in a BoNT/B substrate of the invention.

TABLE 4

Cleavage of VAMP[a,b]



[a]= Sequence corrected in position 93 (f > s).
[b]= Sequence corrected in position 68 (t > s).

The invention also provides a botulinum toxin serotype C1 (BoNT/C1) substrate containing a donor fluorophore; an acceptor having an absorbance spectrum overlapping the emission spectrum of the donor fluorophore; and a BoNT/C1 recognition sequence that includes a cleavage site, where the cleavage site intervenes between the donor fluorophore and the acceptor and where, under the appropriate conditions, resonance energy transfer is exhibited between the donor fluorophore and the acceptor. A BoNT/C1 substrate of the invention can have, for example, at least six consecutive residues of syntaxin, the six consecutive residues including Lys-Ala, or a peptidomimetic thereof. For example, a BoNT/C1 substrate of the invention can have at least six consecutive residues of human syntaxin, the six consecutive residues including Lys_{253}-Ala_{254}, or a peptidomimetic thereof. In one embodiment, a BoNT/C1 substrate contains the amino acid sequence Asp-Thr-Lys-Lys-Ala-Val-Lys-Tyr (SEQ ID NO: 5), or a peptidomimetic thereof.

A BoNT/C1 substrate of the invention also can contain, for example, at least six consecutive residues of SNAP-25, where the six consecutive residues include Arg-Ala, or a peptidomimetic thereof. Such a BoNT/C1 substrate can have, for example, at least six consecutive residues of human SNAP-25, the six consecutive residues including Arg_{198}-Ala_{199}, or a peptidomimetic thereof. An exemplary BoNT/C1 substrate contains residues 93 to 202 of human SNAP-25 (SEQ ID NO: 2), or a peptidomimetic thereof. As for all the clostridial toxin substrates of the invention, a

ping the emission spectrum of the donor fluorophore; and a BoNT/D recognition sequence that includes a cleavage site, where the cleavage site intervenes between the donor fluorophore and the acceptor and where, under the appropriate conditions, resonance energy transfer is exhibited between the donor fluorophore and the acceptor. A BoNT/D substrate of the invention can have, for example, at least six consecutive residues of VAMP, the six consecutive residues including Lys-Leu, or a peptidomimetic thereof. In one embodiment, a BoNT/D substrate contains at least six consecutive residues of human VAMP, the six consecutive residues including Lys_{59}-Leu_{60}, or a peptidomimetic thereof. In another embodiment, a BoNT/D substrate of the invention contains the amino acid sequence Arg-Asp-Gln-Lys-Leu-Ser-Glu-Leu (SEQ ID NO: 6), or a peptidomimetic thereof. In a further embodiment, a BoNT/D substrate of the invention includes residues 27 to 116 of rat VAMP-2 (SEQ ID NO: 7), or a peptidomimetic thereof. It is understood that a variety of donor fluorophore-acceptor combinations are useful in a BoNT/D substrate of the invention; such donor fluorophore-acceptor pairs include, but are not limited to, fluorescein-tetramethylrhodamine; DABCYL-EDANS; and Alexa Fluor® 488-QSY® 7. Additional exemplary donor fluorophores and acceptors useful in a BoNT/D substrate of the invention are provided herein below.

The term "botulinum toxin serotype D recognition sequence" is synonymous with "BoNT/D recognition sequence" and means a scissile bond together with adjacent or non-adjacent recognition elements sufficient for detectable proteolysis at the scissile bond by a BoNT/D under appropriate conditions. A scissile bond cleaved by BoNT/D can be, for example, Lys-Leu.

A variety of BoNT/D recognition sequences are well known in the art or can be defined by routine methods. A BoNT/D recognition sequence can include, for example, residues 27 to 116; residues 37 to 116; residues 1 to 86; residues 1 to 76; or residues 1 to 69 of rat VAMP-2 (SEQ ID NO: 7; Yamasaki et al., *J. Biol. Chem.* 269:12764–12772 (1994)). Thus, a BoNT/D recognition sequence can include, for example, residues 27 to 69 or residues 37 to 69 of rat VAMP-2 (SEQ ID NO: 7). If desired, a similar BoNT/D recognition sequence can be prepared from a corresponding (homologous) segment of another BoNT/D-sensitive VAMP isoform or homolog such as human VAMP-1 or human VAMP-2.

A BoNT/D recognition sequence can correspond to a segment of a protein that is sensitive to cleavage by botulinum toxin serotype D, or can be substantially similar to a segment of a BoNT/D-sensitive protein. As shown in Table 5, a variety of naturally occurring proteins sensitive to cleavage by BoNT/D are known in the art and include, for example, human, mouse and bovine VAMP-1 and VAMP-2; rat VAMP-1 and VAMP-2; rat cellubrevin; chicken VAMP-1 and VAMP-2; Torpedo VAMP-1; Aplysia VAMP; squid VAMP; *Drosophila* syb and n-syb; and leech VAMP. Thus, a BoNT/D recognition sequence useful in a BoNT/D substrate of the invention can correspond, for example, to a segment of human VAMP-1 or VAMP-2, mouse VAMP-1 or VAMP-2, bovine VAMP-1 or VAMP-2, rat VAMP-1 or VAMP-2, rat cellubrevin, chicken VAMP-1 or VAMP-2, Torpedo VAMP-1, Aplysia VAMP, squid VAMP, *Drosophila* syb or n-syb, leech VAMP, or another naturally occurring protein sensitive to cleavage by BoNT/D. Furthermore, as shown in Table 5 above, comparison of native VAMP amino acid sequences cleaved by BoNT/D reveals significant sequence variability (see, also, FIG. **6**), indicating that a variety of amino acid substitutions and modifications relative to a naturally occurring BoNT/D-sensitive VAMP sequence can be tolerated in a BoNT/D substrate of the invention.

The present invention additionally provides a botulinum toxin serotype E (BoNT/E) substrate containing a donor fluorophore; an acceptor having an absorbance spectrum overlapping the emission spectrum of the donor fluorophore; and a BoNT/E recognition sequence that includes a cleavage site, where the cleavage site intervenes between the donor fluorophore and the acceptor and where, under the appropriate conditions, resonance energy transfer is exhibited between the donor fluorophore and the acceptor. A BoNT/E substrate can contain, for example, at least six consecutive residues of SNAP-25, the six consecutive residues including Arg-Ile, or a peptidomimetic thereof. Such a BoNT/E substrate can have, for example, at least six consecutive residues of human SNAP-25, the six consecutive residues including Arg_{180}-Ile_{181}, or a peptidomimetic thereof. In one embodiment, a BoNT/E substrate includes the amino acid sequence Gln-Ile-Asp-Arg-Ile-Met-Glu-Lys (SEQ ID NO: 8), or a peptidomimetic thereof. In another embodiment, a BoNT/E substrate includes residues 156 to 186 of human SNAP-25 (SEQ ID NO: 2), or a peptidomimetic thereof. A variety of donor fluorophore-acceptor combinations are useful in a BoNT/E substrate of the invention. These donor fluorophore-acceptor combinations include, without limitation, fluorescein-tetramethylrhodamine, DABCYL-EDANS, Alexa Fluor® 488-QSY® 7, and additional donor fluorophores and acceptors described further below.

As used herein, the term "botulinum toxin serotype E recognition sequence" is synonymous with "BoNT/E recognition sequence" and means a scissile bond together with adjacent or non-adjacent recognition elements sufficient for detectable proteolysis at the scissile bond by a BoNT/E under appropriate conditions. A scissile bond cleaved by BoNT/E can be, for example, Arg-Ile.

One skilled in the art appreciates that a BoNT/E recognition sequence can correspond to a segment of a protein that is sensitive to cleavage by botulinum toxin serotype E, or can be substantially similar to a segment of a BoNT/E-sensitive protein. A variety of naturally occurring proteins sensitive to cleavage by BoNT/E are known in the art and include, for example, human, mouse and rat SNAP-25; mouse SNAP-23; chicken SNAP-25; goldfish SNAP-25A and SNAP-25B; zebrafish SNAP-25; *C. elegans* SNAP-25; and leech SNAP-25 (see Table 2). Thus, a BoNT/E recognition sequence useful in a BoNT/E substrate of the invention can correspond, for example, to a segment of human SNAP-25, mouse SNAP-25, rat SNAP-25, mouse SNAP-23, chicken SNAP-25, goldfish SNAP-25A or 25B, *C. elegans* SNAP-25, leech SNAP-25, or another naturally occurring protein sensitive to cleavage by BoNT/E. Furthermore, as shown in Table 2 and FIG. **5** above, comparison of native SNAP-23 and SNAP-25 amino acid sequences cleaved by BoNT/E reveals that such sequences are not absolutely conserved, indicating that a variety of amino acid substitutions and modifications relative to a naturally occurring BoNT/E-sensitive SNAP-23 or SNAP-25 sequence can be tolerated in a BoNT/E substrate of the invention.

The invention also provides a botulinum serotype A/E (BoNT/A/E) substrate containing (a) a donor fluorophore; (b) an acceptor having an absorbance spectrum overlapping the emission spectrum of the donor fluorophore; and (c) a BONT A or BoNT/E recognition sequence containing a cleavage site, where the cleavage site intervenes between the donor fluorophore and the acceptor and where, under the appropriate conditions, resonance energy transfer is exhib-

ited between the donor fluorophore and the acceptor. As used herein, the term "botulinum serotype A/E substrate" or "BoNT/A/E substrate" or "A/E substrate" means a substrate that is susceptible to cleavage either by a botulinum serotype A toxin or a botulinum serotype E toxin. Such a botulinum serotype A/E substrate also can be susceptible to cleavage by both the BoNT/A and BoNT/E toxins. Any of the BoNT/A or BoNT/E recognition sequences described herein or known in the art are useful in a BoNT/A/E substrate of the invention.

Further provided by the invention is a botulinum toxin serotype F (BoNT/F) substrate containing a donor fluorophore; an acceptor having an absorbance spectrum overlapping the emission spectrum of the donor fluorophore; and a BoNT/F recognition sequence that includes a cleavage site, where the cleavage site intervenes between the donor fluorophore and the acceptor and where, under the appropriate conditions, resonance energy transfer is exhibited between the donor fluorophore and the acceptor. Such a BoNT/F substrate can have, for example, at least six consecutive residues of VAMP, the six consecutive residues including Gln-Lys, or a peptidomimetic thereof. In one embodiment, a BoNT/F substrate has at least six consecutive residues of human VAMP, the six consecutive residues including Gln_{58}-Lys_{59}, or a peptidomimetic thereof. In another embodiment, a BoNT/F substrate of the invention includes residues 27 to 116 of rat VAMP-2 (SEQ ID NO: 7), or a peptidomimetic thereof. In a further embodiment, a BoNT/F substrate includes the amino acid sequence Glu-Arg-Asp-Gln-Lys-Leu-Ser-Glu (SEQ ID NO: 9), or a peptidomimetic thereof. Those skilled in the art of fluorescence resonance energy transfer understand that a variety of donor fluorophore-acceptor combinations are useful in a BoNT/F substrate of the invention. Non-limiting examples of donor fluorophore-acceptor pairs useful in a BoNT/F substrate of the invention include fluorescein-tetramethylrhodamine, DABCYL-EDANS, Alexa Fluor® 488-QSY® 7, as well as additional donor fluorophore-acceptors combinations described further below.

The term "botulinum toxin serotype F recognition sequence," as used herein, is synonymous with "BoNT/F recognition sequence" and means a scissile bond together with adjacent or non-adjacent recognition elements sufficient for detectable proteolysis at the scissile bond by a BoNT/F under appropriate conditions. A scissile bond cleaved by BoNT/F can be, for example, Gln-Lys.

A variety of BoNT/F recognition sequences are well known in the art or can be defined by routine methods. A BoNT/F recognition sequence can include, for example, residues 27 to 116; residues 37 to 116; residues 1 to 86; residues 1 to 76; or residues 1 to 69 of rat VAMP-2 ((SEQ ID NO: 7; Yamasaki et al., supra, 1994). A BoNT/F recognition sequence also can include, for example, residues 27 to 69 or residues 37 to 69 of rat VAMP-2 (SEQ ID NO: 7). It is understood that a similar BoNT/F recognition sequence can be prepared, if desired, from a corresponding (homologous) segment of another BoNT/F-sensitive VAMP isoform or homolog such as human VAMP-1 or human VAMP-2.

A BoNT/F recognition sequence can correspond to a segment of a protein that is sensitive to cleavage by botulinum toxin serotype F, or can be substantially similar to a segment of a BoNT/F-sensitive protein. A variety of naturally occurring proteins sensitive to cleavage by BoNT/F are known in the art and include, for example, human, mouse and bovine VAMP-1 and VAMP-2; rat VAMP-1 and VAMP-2; rat cellubrevin; chicken VAMP-1 and VAMP-2; Torpedo VAMP-1; Aplysia VAMP; *Drosophila* syb; and leech VAMP (see Table 5). Thus, a BoNT/F recognition sequence useful in a BoNT/F substrate of the invention can correspond, for example, to a segment of human VAMP-1 or VAMP-2, mouse VAMP-1 or VAMP-2, bovine VAMP-1 or VAMP-2, rat VAMP-1 or VAMP-2, rat cellubrevin, chicken VAMP-1 or VAMP-2, Torpedo VAMP-1, Aplysia VAMP, *Drosophila* syb, leech VAMP, or another naturally occurring protein sensitive to cleavage by BoNT/F. Furthermore, as shown in Table 5 above, comparison of native VAMP amino acid sequences cleaved by BoNT/F reveals that such sequences are not absolutely conserved (see, also, FIG. **6**), indicating that a variety of amino acid substitutions and modifications relative to a naturally occurring BoNT/F-sensitive VAMP sequence can be tolerated in a BoNT/F substrate of the invention.

The present invention also provides a botulinum toxin serotype G (BoNT/G) substrate containing a donor fluorophore; an acceptor having an absorbance spectrum overlapping the emission spectrum of the donor fluorophore; and a BoNT/G recognition sequence that includes a cleavage site, where the cleavage site intervenes between the donor fluorophore and the acceptor and where, under the appropriate conditions, resonance energy transfer is exhibited between the donor fluorophore and the acceptor. A BoNT/G substrate can have, for example, at least six consecutive residues of VAMP, the six consecutive residues including Ala-Ala, or a peptidomimetic thereof. Such a BoNT/G substrate can have, for example, at least six consecutive residues of human VAMP, the six consecutive residues including Ala_{83}-Ala_{84}, or a peptidomimetic thereof. In one embodiment, a BoNT/G substrate contains the amino acid sequence Glu-Thr-Ser-Ala-Ala-Lys-Leu-Lys (SEQ ID NO: 10), or a peptidomimetic thereof. As discussed above in regard to other clostridial toxin substrates, a variety of donor fluorophore-acceptor combinations are useful in a BoNT/G substrate of the invention including for example, fluorescein-tetramethylrhodamine, DABCYL-EDANS, Alexa Fluor® 488-QSY® 7, and other donor fluorophore-acceptor combinations disclosed herein below or well known in the art.

As used herein, the term "botulinum toxin serotype G recognition sequence" is synonymous with "BoNT/G recognition sequence" and means a scissile bond together with adjacent or non-adjacent recognition elements sufficient for detectable proteolysis at the scissile bond by a BoNT/G under appropriate conditions. A scissile bond cleaved by BoNT/G can be, for example, Ala-Ala.

A BoNT/G recognition sequence can correspond to a segment of a protein that is sensitive to cleavage by botulinum toxin serotype G, or can be substantially similar to such a BoNT/G-sensitive segment. As illustration in Table 5 above, a variety of naturally occurring proteins sensitive to cleavage by BoNT/G are known in the art and include, for example, human, mouse and bovine VAMP-1 and VAMP-2; rat VAMP-1 and VAMP-2; rat cellubrevin; chicken VAMP-1 and VAMP-2; and Torpedo VAMP-1. Thus, a BoNT/G recognition sequence useful in a BoNT/G substrate of the invention can correspond, for example, to a segment of human VAMP-1 or VAMP-2, mouse VAMP-1 or VAMP-2, bovine VAMP-1 or VAMP-2, rat VAMP-1 or VAMP-2, rat cellubrevin, chicken VAMP-1 or VAMP-2, Torpedo VAMP-1, or another naturally occurring protein sensitive to cleavage by BoNT/G. Furthermore, as shown in Table 5 above, comparison of native VAMP amino acid sequences cleaved by BoNT/G reveals that such sequences are not absolutely conserved (see, also, FIG. **6**), indicating that a variety of amino acid substitutions and modifications relative to a

TABLE 6-continued

Donor fluorophore	Acceptor	R_o (Å)	Reference
FMA	FMA	37	Dissing et al., Biochim. Biophys. Acta 553: 66–83 (1979)
PM	DMAMS	38	Lin and Dowben, J. Biol. Chem. 258: 5142–5150 (1983)
mBBR	FITC	38	Tompa and Batke, Biochem. Int. 20: 487–494 (1990)
mBBR	DABM	38	Kasprzak et al., Biochemistry 27: 4512–4523 (1988)
ϵA	NBD	38	Miki and Iio, Biochim. Biophys. Acta 790: 201–207 (1984)
Pyrene	Coumarin	39	Borochov-Neori and Montal, supra, 1989
IPM	FNAI	39	Peerce and Wright, supra, 1986
IAEDANS	DABM	40	Tao et al. Biochemistry 22: 3059–3066 (1983)
IAEDANS	TNP-ATP	40	Tao et al., supra, 1983
ϵ-A	IANBD	40	Miki and Wahl, Biochim. Biophys. Acta 786: 188–196 (1984)
NBD	SRH	40–74	Wolf et al., Biochemistry 31: 2865–2873 (1992)
ISA	TNP	42	Jacobson and Colman, Biochemistry 23: 3789–3799 (1984)
Dansyl	ODR	43	Lu et al., J. Biol. Chem. 264: 12956–12962 (1989)
DANZ	IAF	44–49	Cheung et al., Biochemistry 21: 5135–5142 (1983)
FNAI	EITC	45	Peerce and Wright, supra, 1986
NBD	LRH	45–70	Wolf et al., supra, 1992
IAF	EIA	46	Taylor et al., supra, 1981
FITC	ENAI	46	Peerce and Wright, supra, 1986
Proflavin	ETSC	46	Robbins et al., Biochemistry 20: 5301–5309 (1981)
CPM	TNP-ATP	46	Snyder and Hammes, supra, 1985
IAEDANS	IAF	46–56	Franzen, supra, 1985; Grossman, supra, 1990
CPM	Fluorescein	47	Thielen et al., Biochemistry 23: 6668–6674 (1984)
IAEDANS	FITC	49	Jona et al., Biochim. Biophys. Acta 1028: 183–199 (1990); Birmachu et al., Biochemistry 28: 3940–3947 (1989)
IAF	TMR	50	Shahrokh et al., J. Biol. Chem. 266: 12082–12089 (1991)
CF	TR	51	Johnson et al., supra, 1993
CPM	TRS	51	Odom et al., supra, 1984
ϵ-A	TNP-ATP	51	dos Remedios and Cooke, supra, 1984
CPM	FM	52	Odom et al., supra, 1984
LY	EM	53	Shapiro et al., J. Biol. Chem. 266: 17276–17285 (1991)
FITC	EITC	54	Carraway et al., J. Biol. Chem. 264: 8699–8707 (1989)
IAEDANS	DiO-C_{14}	57	Shahrokh et al., supra, 1991
IAF	ErITC	58	Amler et al., supra, 1992
FITC	EM	60	Kosk-Kosicka et al., J. Biol. Chem. 264: 19495–19499 (1989)
FITC	ETSC	61–64	Robbins et al., supra, 1981
FITC	ErITC	62	Amler et al., supra, 1992
BPE	CY5	72	Ozinskas et al., Anal. Biochem. 213: 264–270 (1993)
Fluorescein	Fluorescein	44	—
BODIBY FL	BODIPY FL	57	—

ANAI, 2-anthracence N-acetylimidazole;
BPE, B-phycoerythrin;
CF, carboxyfluorescein succinimidyl ester;
CPM, 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin;
CY5, carboxymethylindocyanine-N-hydroxysuccinimidyl ester;
diI-C_{18}, 1,1'-dioctadecyl-3-3,3,3',3'-tetramethyl-indocarbocyanine;
diO-C_{14}, 3,3'-ditetradecyloxacarbocyanine;
DABM, 4-dimethylaminophenylazo-phenyl-4'-maleimide;
DACM, (7-(dimethylamino)coumarin-4-yl)-acetyl;
DANZ, dansylaziridine;
DDPM, N-(4-dimethylamino-3,5-dinitrophenyl)maleimide;
DMAMS, dimethylamino-4-maleimidostilbene;
DSMN, N-(2,5'-dimethoxystiben-4-yl)-maleimide;
DNP, 2,4 dinitrophneyl;
ϵ-A, 1,N^6-ethenoadenosine;
EIA, 5-(iodoacetetamido) eosin;
EITC, eosin-5-isothiocyanate;
ENAI, eosin N-acETYLIMIDAZOLE;
EM, eosin maleimide;
ErITC, erythrosin-5'-isothiocyanate;
ETSC, eosin thiosemicarazide;
F_2DNB, 1,5-difluro-2,4'-dinitrobenzene;
F_2DPS, 4,4'-difluoro-3,3'-dinitrophenylsulfone;
FITC, fluorescein thiosemicarbazide;
IAANS, 2-(4'-iodoacetamido)anilino)napthalene-6-sulfonic acid;
IAEDANS, 5-(2-((iodoacetyl)amino)ethyl)amino) napthlene 1-sulfonic acid;
IAF, 5-iodoacetamidofluorescein;
IANBD, N-((2-(iodoacetoxy)ethyl)-N-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole;
IPM, 3(4-isothiocyanatophenyl)7-diethyl-4-amino-4-methylcoumarin;
ISA, 4-(iodoacetamido)salicylic acid;
LRH, lissaminerhodamine;
LY, Lucifer yellow;
mBBR, monobromobiamane;
MNA, (2-methoxy-1-naphthyl)-methyl;
NAA, 2-napthoxyacetic acid;
NBD, 7-nirto-2,1,3-benzoxadiazol-4-yl;
NCP, N-cyclohexyl-N'-(1-pyrenyl) carbodiimide;
ODR, octadecylrhodamine;
PM, N-(1-pyrene)-maleimide;
SRH, sulforhodamine;
TMR, tetramethylrhodamine;
TNP, trinitrophenyl; and
TR, Texas Red

An aromatic amino acid such as tryptophan or tyrosine also can be a donor fluorophore useful in a clostridial toxin substrate of the invention. Exemplary donor fluorophore-acceptor pairs in which tryptophan or tyrosine is the donor fluorophore and relevant Förster distances are shown in Table 7 below. Modified amino acids also can be useful as donor fluorophores or acceptors in a clostridial toxin sub-

strate of the invention. Such fluorescent or quenching modified amino acids are known in the art and include, for example, the fluorescent amino acid L-pyrenylalanine (Pya) and the non-fluorescent acceptor p-nitrophenylalanine (Nop), as described, for example, in Anne et al., *Analytical Biochem.* 291:253–261 (2001).

TABLE 7

Förster Distances Using Trp as a Donor

Donor	Acceptor	R_o (Å)	Reference
Trp	Ru(III) $(NH_3)_5$	12–16	Recchia et al., Biochim. Biophys. Acta 702: 105–111 (1982)
Trp	Nitrobenzoyl	16	Wiczk et al., J. Fluo 1: 273–286 (1991)
Trp	Dansyl	21	Steinberg, Annu. Rev. Biochem. 40: 83–114 (1971)
Trp	IAEDANS	22	Matsumoto and Hammes, Biochemistry 14: 214–224 (1975)
Trp	ANS	23	Conrad and Brand, Biochemistry 7: 777–787 (1968)
Trp	Anthroyloxy	24	Wiczk et al., supra, 1991
Trp	TNB	24	Wu and Brand, Biochemistry 31: 7939–7947 (1992)
Trp	Anthroyl	25	Burgun et al., Arch. Biochem. Biophys. 286: 394–401 (1991)
Trp	Tyr-NO_2	26	Steiner et al., J. Fluo. 1: 15–22 (1991)
Trp	Pyrene	28	Vekshin, Mol. Biol. 17: 827–832 (1983)
Trp	Heme	29	Ladokhin et al., Proc. SPIE 1640: 562–569 (1992)
Trp	NBS	30	Wiczk et al., supra, 1991
Trp	DNBS	33	Wiczk et al., supra, 1991
Trp	DPH	40	Le Doan et al., Biochim. Biophys. Acta 735: 259–270 (1983)

In view of the above, it is understood that a variety of donor fluorophore/acceptor pairs can be useful in a clostridial toxin substrate of the invention. A donor fluorophore-acceptor pair useful in the invention can be, for example, the donor fluorophore fluorescein in combination with ROX (6-carboxy-X-rhodamine; Applied Biosystems Division of Perkin-Elmer Corporation; Foster City, Calif.); TAMRA (N,N,N',N'-tetramethyl-6-carboxy-rhodamine; Applied Biosystems); rhodamine; texas red or eosin. A donor fluorophore-acceptor pair useful in the invention also can be, for example, the donor fluorophore cascade blue with fluorescein as an acceptor; the donor fluorophore BODIPY® 530/550 (4,4-difluoro-5,7-diphenyl-4-bora-3a, 4a-diaza-S-indacene) in combination with BODIPY® 542/563 (4,4-difluoro-5-p-methoxyphenyl-4-bora-3a,4a-diaza-S-indacene) as an acceptor; or BODIPY® 542/563 (4,4-difluoro-5-p-methoxyphenyl-4-bora-3a,4a-diaza-S-indacene) in combination with BODIPY® 564/570 (4,4-difluoro-5-styryl-4-bora-3a,4a-diaza-S-indacene) as an acceptor. The numbers following the name BODIPY® reflect the excitation and emission maxima of the molecule; BODIPY® compounds are commercially available from Molecular Probes (Eugene Oreg.).

In one embodiment, the donor fluorophore is fluorescein. In a further embodiment, a clostridial toxin substrate of the invention contains a fluorescein as the donor fluorophore and tetramethylrhodamine as the acceptor. Such a substrate can be excited in the range of 480 to 505 nm, for example, at 488 nm or 492 nm, and emission detected at 520 nm (λ_{em} fluorescein), 585 nm (λ_{em} tetramethylrhodamine), or both. Prior to cleavage of the substrate at the clostridial toxin cleavage site, the tetramethylrhodamine emission intensity is greater than that of fluorescein; substrate cleavage results in a change in the ratio of fluorescein to tetramethylrhodamine intensity. Cleavage generally results in fluorescein becoming the dominant emitting fluorophore. Methods for preparing proteins and peptides containing fluorescein and tetramethylrhodamine are well known in the art (see, for example, Matsumoto et al., *Bioorganic & Medicinal Chemistry Letters* 10:1857–1861 (2000)).

A donor fluorophore useful in a substrate of the invention also can be, for example, EDANS (λ_{Ab} 340 nM, λ_{Em} 490 nm), which can be combined with an acceptor such as DABCYL. Where DABCYL and EDANS are combined in a clostridial toxin substrate of the invention, energy is transferred from the EDANS donor fluorophore to the DABCYL acceptor in the intact substrate, resulting in quenching of EDANS emission fluorescence. Upon cleavage at the toxin cleavage site, fluorescence of the cleaved EDANS product is increased and can be restored, for example, to the free donor fluorophore level. Efficient fluorescence quenching in the intact substrate occurs as a result of favorable energetic overlap of the EDANS emission spectrum and the DABCYL absorbance spectrum, and the relatively long excited state lifetime of the EDANS donor fluorophore (Wang et al., *Tetrahedron Lett.* 31:6493–6496 (1991); Holskin et al., *Anal. Biochem.* 226:148–155 (1995); and Wang et al., *Anal. Biochem.* 210:351–359 (1993)).

Dansyl (DNS or 5-dimethylaminonaphthalene-1-sulfonyl) also can be a useful as a donor fluorophore or acceptor in a substrate of the invention. In one embodiment, a clostridial toxin substrate of the invention contains dansyl as the donor fluorophore; a dansyl donor can be combined, for example, with a nitrophenyl residue acceptor such as Phe (pNO2), which acts as a quencher when in proximity to the dansyl donor fluorophore. Substrates containing a dansyl donor fluorophore, for example, in combination with a nitrophenyl residue can be prepared as described, for example, in Florentin et al., *Anal. Biochem.* 141:62–69 (1984) or Goudreau et al., *Anal. Biochem.* 219:87–95 (1994). In another embodiment, a clostridial toxin substrate contains dansyl as the acceptor. A dansyl acceptor can act as a quencher when combined, for example, with a donor fluorophore such as Trp (λ_{ex} 290 nm, λ_{em} 360 nm). In a substrate containing Trp and dansyl, Trp fluorescence can be quenched 60% by energy transfer to the dansyl group, and this quenching can be significantly reduced or abolished in the presence of toxin protease activity at the toxin cleavage site (see, for example, Geoghegan et al., *FEBS Letters* 262:119–122 (1990)).

It is understood that donor-acceptor pairs having well-separated emission maxima can be useful in the substrates and methods of the invention; well-separated emission maxima allow altered acceptor emission to be detected without donor emission contamination. A donor fluorophore, or acceptor, or both, can emit, for example, in the far-red, for example, greater than 650 nm. Such far-red emitting donor fluorophores and acceptors include cyanine dyes such as Cy5, Cy5.5 and Cy7 (Selvin, supra, 2000). In one embodiment, the invention provides a clostridial toxin substrate containing Cy3 and Cy5 as the donor fluorophore-acceptor pair; Cy3 emits maximally as 570 nm and Cy5 emits maximally at 670 nm. Such cyanine dyes can be prepared by straightforward synthesis, as described, for example, in Gruber et al., *Bioconj. Chem.* 11:161–166 (2000).

fluorophore and acceptor can be selected and positioned, for example, so as to minimize the disruption of bonded and non-bonded interactions that are important for binding, and to minimize steric hindrance. In addition, the spatial distance between the acceptor and donor fluorophore generally is limited to achieve efficient energy transfer from the donor fluorophore to the acceptor.

As discussed above, efficiency of energy transfer from donor fluorophore to acceptor is dependent, in part, on the spatial separation of the donor fluorophore and acceptor molecules. As the distance between the donor fluorophore and acceptor increases, there is less energy transfer to the acceptor, and the donor fluorescence signal therefore increases, even prior to cleavage. The overall increase in fluorescence yield of the donor fluorophore, upon cleavage of the substrate, is dependent upon many factors, including the separation distance between the donor fluorophore and acceptor in the substrate, the spectral overlap between donor fluorophore and acceptor, and the concentration of substrate used in an assay. One skilled in the art understands that, as the concentration of substrate increases, intermolecular quenching of the donor, even after proteolytic cleavage, can become a factor. This phenomenon is denoted the "inner filter effect" (see below).

The Förster distance, which is the separation between a donor fluorophore and an acceptor for 50% energy transfer, represents a spatial separation between donor fluorophore and acceptor that provides a good sensitivity. For peptide substrates, adjacent residues are separated by a distance of approximately 3.6 Å in the most extended conformation. For example, the calculated Förster distance for a fluorescein/tetramethylrhodamine pair is 55 Å, which would represent a spatial separation between fluorescein and tetramethylrhodamine of about 15 residues in the most extended conformation. Because peptides and peptidomimetics in solution rarely have a fully extended conformation, donor fluorophores and acceptors can be more widely separated than expected based on a calculation performed using 3.6 Å per residue and still remain within the Förster distance.

Förster theory is based on very weak interactions between donor fluorophore and acceptor; spectroscopic properties such as absorption of one fluorophore should not be altered in the presence of the other, defining the shortest distance range over which the theory is valid. It is understood that, for many donor fluorophore-acceptor pairs, Förster theory is valid when donor fluorophores and acceptors are separated by about 10 Å to 100 Å. However, for particular donor fluorophore-acceptor pairs, Förster theory is valid below 10 Å as determined by subpicosecond techniques (Kaschke and Ernsting, *Ultrafast Phenomenon in Spectroscopy* (Klose and Wilhelmi (Eds.)) Springer-Verlag, Berlin 1990.

Thus, in one embodiment, the invention provides a clostridial toxin substrate in which a donor fluorophore is separated from an acceptor by a distance of at most 100 Å. In other embodiments, the invention provides a clostridial toxin substrate in which a donor fluorophore is separated from an acceptor by a distance of at most 90 Å, 80 Å, 70 Å, 60 Å, 50 Å, 40 Å, 30 Åor 20 Å. In further embodiments, the invention provides a clostridial toxin substrate in which a donor fluorophore is separated from an acceptor by a distance of 10 Å to 100 Å, 10 Å to 80 Å, 10 Å to 60 Å, 10 Å to 40 Å, 10 Å to 20 Å, 20 Å to 100 Å, 20 Å to 80 Å, 20 Å to 60 Å, 20 Å to 40 Å, 40 Å to 100 Å, 40 Å to 80 Å or 40 Å to 60 Å.

One skilled in the art understands that a clostridial toxin substrate of the invention can be designed to optimize the efficiency of FRET as well as the ability to detect protease activity. One skilled in the art understands that a donor fluorophore can be selected, if desired, with a high quantum yield, and acceptor can be selected, if desired, with a high extinction coefficient to maximize the Förster distance. One skilled in the art further understands that fluorescence arising from direct excitation of an acceptor can be difficult to distinguish from fluorescence resulting from resonance energy transfer. Thus, it is recognized that a donor fluorophore and acceptor can be selected which have relatively little overlap of their excitation spectra such that the donor can be excited at a wavelength that does not result in direct excitation of the acceptor. It further is recognized that a clostridial toxin substrate of the invention can be designed so that the emission spectra of the donor fluorophore and acceptor overlap relatively little such that the two emissions can be readily distinguished. If desired, an acceptor having a high fluorescence quantum yield can be selected; such an acceptor is preferred if acceptor fluorescence emission is to be detected as the sole indicator of clostridial toxin protease activity, or as part of an emission ratio (see below).

It is understood that the donor fluorophore, acceptor, or both, can be located within the active site cavity of botulinum or tetanus toxin holoenzyme. One skilled in the art understands that, if desired, a clostridial toxin substrate can be designed such that, when bound by toxin, the donor fluorophore, acceptor, or both, is excluded from the active site cavity of toxin holoenzyme. Thus, in one embodiment, the invention provides a botulinum toxin substrate or tetanus toxin substrate in which, when bound by toxin, the donor fluorophore, acceptor, or both, is excluded from the active site cavity of clostridial toxin holoenzyme. The invention provides, for example, a BoNT/A, BoNT/B, BoNT/C1, BoNT/D, BoNT/E, BoNT/F or BoNT/G substrate in which, when bound by toxin, the donor fluorophore, acceptor, or both, is excluded from the active site cavity of toxin holoenzyme. In one embodiment, the invention provides a BoNT/A substrate containing at least six residues of human SNAP-25, where the six residues include Gln_{197}-Arg_{198}, in which the donor fluorophore, acceptor, or both, are not positioned between residues Arg_{191} to Met_{202}, which can be within the active site cavity of BoNT/A holoenzyme. In another embodiment, the invention provides a BoNT/B substrate containing at least six residues of VAMP-2, where the six residues include Gln_{76}-Phe_{77}, in which the donor fluorophore, acceptor, or both, are not positioned between residues Leu_{70} to Ala_{81} of VAMP-2, which are within the active site cavity of BoNT/B holoenzyme.

In a complex of a VAMP substrate and the light chain of BoNT/B (LC/B), nearly all VAMP residues with side chains containing hydrogen bond acceptors or donors were hydrogen bonded with the LC/B. Thus, it is understood that a clostridial toxin substrate of the invention can be prepared, if desired, in which the potential for hydrogen bonding, for example, by Ser, Thr, Tyr, Asp, Glu, Asn or Gln residues is not diminished in the clostridial toxin substrate as compared to a native protein sensitive to cleavage by the toxin. Thus, in particular embodiments, the present invention provides a clostridial toxin substrate in which the potential for hydrogen-bonding is not diminished in the clostridial toxin substrate as compared to a native protein sensitive to cleavage by the corresponding botulinum or tetanus toxin.

It is understood that, in addition to a donor fluorophore, acceptor and clostridial toxin recognition sequence, a clostridial toxin substrate of the invention can include, if desired, one or more additional components. As an example, a flexible spacer sequence such as GGGGS (SEQ ID NO: 84) can be included in a clostridial toxin substrate of the

invention. A substrate further also can include, without limitation, one or more of the following: an affinity tag such as HIS6, biotin, or an epitope such as FLAG, hemagluttinin (HA), c-myc, or AU1; an immunoglobulin hinge region; an N-hydroxysuccinimide linker; a peptide or peptidomimetic hairpin turn; or a hydrophilic sequence, or another component or sequence that promotes the solubility or stability of the clostridial toxin substrate.

Methods for modifying proteins, peptides and peptidomimetics to contain a donor fluorophore or acceptor are well known in the art (Fairclough and Cantor, *Methods Enzymol.* 48:347–379 (1978); Glaser et al., *Chemical Modification of Proteins* Elsevier Biochemical Press, Amsterdam (1975); Haugland, *Excited States of Biopolymers* (Steiner Ed.) pp. 29–58, Plenum Press, New York (1983); Means and Feeney, *Bioconjugate Chem.* 1:2–12 (1990); Matthews et al., *Methods Enzymol.* 208:468–496 (1991); Lundblad, *Chemical Reagents for Protein Modification* 2nd Ed., CRC Press, Boca Ratan, Fla. (1991); Haugland, supra, 1996). A variety of groups can be used to couple a donor fluorophore or acceptor, for example, to a peptide or peptidomimetic containing a clostridial toxin recognition sequence. A thiol group, for example, can be used to couple a donor fluorophore or acceptor to the desired position in a peptide or peptidomimetic to produce a clostridial toxin substrate of the invention. Haloacetyl and maleimide labeling reagents also can be used to couple donor fluorophores or acceptors in preparing a substrate of the invention (see, for example, Wu and Brand, supra, 1994.

Donor fluorophores and acceptors including proteins such as GFP and allophycocyanin (APC) can be attached to a clostridial toxin recognition sequence by a variety of means. A donor fluorophore or acceptor can be attached by chemical means via a cross-linker moiety. Cross-linkers are well known in the art, including homo-or hetero-bifunctional cross-linkers such as BMH and SPDP. Where the donor fluorophore or acceptor is a protein, well known chemical methods for specifically linking molecules to the amino- or carboxy-terminus of a protein can be employed. See, for example, "Chemical Approaches to Protein Engineering" in *Protein Engineering-A Practical Approach* Rees et al. (Eds) Oxford University Press, 1992.

One skilled in the art understands that contaminating substrates containing only the donor fluorophore can result in high fluorescence background. Such background can be reduced or prevented, for example, by using a relative excess of acceptor to donor fluorophore in preparation of the clostridial toxin substrate.

The present invention also provides kits for determining clostridial toxin protease activity in a sample. The kit contains a clostridial toxin substrate of the invention in a vial or other container. The kit generally also includes instructions for use. In one embodiment, a kit of the invention further includes as a positive control a known amount of the botulinum or tetanus toxin capable of cleaving the clostridial toxin substrate included in the kit. In another embodiment, the kit contains a clostridial toxin substrate of the invention and further includes one or both cleavage products as a positive controls. In a particular embodiment, the kit contains a clostridial toxin substrate of the invention and the corresponding cleavage product that includes the donor fluorophore as a positive control. A kit of the invention optionally can include a container with buffer suitable for clostridial toxin protease activity. A described further herein below, the methods of the invention can be practiced with a combination of clostridial toxin substrates. Thus, in one embodiment, the invention provides a kit for determining clostridial toxin protease activity that includes at least two clostridial toxin substrates of the invention.

The present invention also provides clostridial toxin targets useful for detecting clostridial toxin protease activity. A clostridial toxin target is a polypeptide, peptide or peptidomimetic which contains a donor fluorophore; an acceptor; and a clostridial toxin recognition sequence that includes a cleavage site, where the cleavage site intervenes between the donor fluorophore and the acceptor and where, under the appropriate conditions, energy transfer is exhibited between the donor fluorophore and the acceptor. Energy can be transferred, for example, via collisional energy transfer and does not require that the acceptor have an absorbance spectrum which overlaps the emission spectrum of the donor fluorophore. Such a clostridial toxin target can include, for example, a botulinum toxin recognition sequence. Any of the clostridial toxin recognition sequences disclosed herein are useful in a substrate of the invention also can be useful in a clostridial toxin target of the invention. Selection and positioning of donor fluorophores and acceptors such that collisional energy transfer is exhibited is well known in the art, as described, for example, in Gershkkovich and Kholodovych, *J. Biochem. Biophys. Methods* 33:135–162 (1996).

The present invention also provides methods of determining clostridial toxin protease activity. Such methods are valuable, in part, because they are amenable to rapid screening and do not require separation of cleaved products from uncleaved substrate. Furthermore, the methods of the invention are applicable to crude samples as well as highly purified dichain toxins and further are applicable to clostridial toxin light chains, as described further below. The methods of the invention include the following steps: (a) treating a sample, under conditions suitable for clostridial toxin protease activity, with a clostridial toxin substrate that contains a donor fluorophore, an acceptor having an absorbance spectrum overlapping the emission spectrum of the donor fluorophore, and a clostridial toxin recognition sequence containing a cleavage site, where the cleavage site intervenes between the donor fluorophore and the acceptor and where, under the appropriate conditions, resonance energy transfer is exhibited between the donor fluorophore and the acceptor; (b) exciting the donor fluorophore; and (c) determining resonance energy transfer of the treated substrate relative to a control substrate, where a difference in resonance energy transfer of the treated substrate as compared to the control substrate is indicative of clostridial toxin protease activity. A method of the invention can be practiced with an acceptor which is a fluorophore, or with a non-fluorescent acceptor.

A method of the invention can be used to determine protease activity of any clostridial toxin. In one embodiment, a method of the invention relies on a BoNT/A substrate to determine BoNT/A protease activity. A BoNT/A substrate useful in a method of the invention can be any of the BoNT/A substrates disclosed herein, for example, a BoNT/A substrate containing at least six consecutive residues of SNAP-25, where the six consecutive residues include Gln-Arg. In another embodiment, a method of the invention relies on a BoNT/B substrate to determine BoNT/B protease activity. A BoNT/B substrate useful in a method of the invention can be any of the BoNT/B substrates disclosed herein, for example, a BoNT/B substrate containing at least six consecutive residues of VAMP, where the six consecutive residues include Gln-Phe. A method of the invention also can utilize a BoNT/C1 substrate to determine BoNT/C1 protease activity. A BoNT/C1 substrate

Thr-Arg-Ile-Asp-Glu-Ala-Asn-Gln (SEQ ID NO: 86) and Arg-Ala-Thr-Lys-Met-Leu-Z2-NH_2 (SEQ ID NO: 87) are produced.

Additional FRET substrates also are synthesized: X1-Asp-Ser-Asn-Lys-Thr-Arg-Ile-Asp-Glu-Ala-Asn-Gln-Arg-Ala-Thr-Lys-Met-Leu-Gly-Ser-Gly-Z2-NH_2 (SEQ ID NO: 88); X1-Ala-Asp-Ser-Asn-Lys-Thr-Arg-Ile-Asp-Glu-Ala-Asn-Gln-Arg-Ala-Thr-Lys-Met-Leu-Z2-NH_2 (SEQ ID NO: 89); X1-Ala-Asp-Ser-Asn-Lys-Thr-Arg-Ile-Asp-Glu-Ala-Asn-Gln-Arg-Ala-Thr-Lys-Met-Leu-Gly-Ser-Gly-Z2-NH_2 (SEQ ID NO: 90); X1-Thr-Arg-Ile-Asp-Glu-Ala-Asn-Gln-Arg-Ala-Thr-Lys-Met-Leu-Z2-NH_2 (SEQ ID NO: 91); X1-Thr-Arg-Ile-Asp-Glu-Ala-Asn-Gln-Arg-Ala-Thr-Lys-Met-Leu-Gly-Ser-Gly-Z2-NH_2 (SEQ ID NO: 92); X1-Met-Glu-Lys-Thr-Arg-Ile-Asp-Glu-Ala-Asn-Gln-Arg-Ala-Thr-Lys-Met-Leu-Gly-Ser-Gly-Z2-NH_2 (SEQ ID NO: 93), in each of which X1 is a fluorescein-modified lysine residue and Z2 is a tetramethylrhodamine-modified lysine residue; X3-Thr-Arg-Ile-Asp-Glu-Ala-Asn-Gln-Arg-Ala-Thr-Lys-Met-Leu-Z4-NH_2 (SEQ ID NO: 94), in which X3 is a DABCYL modified lysine residue and Z4 is a EDANS modified glutamate residue; and X3-Thr-Arg-Ile-Asp-Glu-Ala-Asn-Gln-Arg-Ala-Thr-Lys-Met-Leu-Gly-Ser-Gly-Z5-NH_2 (SEQ ID NO: 95), in which X3 is a DABCYL modified lysine residue and Z5 is a EDANS modified lysine residue.

Purified BoNT/A light chain (LC/A) or cellular extract containing LC/A is diluted in assay buffer (0.05 M HEPES (pH 7.4); 1% FBS; 10 μM $ZnCl_2$; and 10 mM DTT). Dichain BoNT/A is incubated with 10 mM dithiothreitol (DTT) for about 30 minutes prior to analysis. Reactions contain various concentrations of LC/A, dichain toxin or formulated BOTOX® product, from 0.1 ng to 10 μg. Toxin is assayed as follows: FRET substrate is added to a final concentration of 10 μM in a final volume of 100 μL assay buffer. The reaction is incubated at 37° C. for 30 minutes, and is subsequently terminated by addition of 50 μL 2M H_2SO_4.

Fluorescence is measured in a fluorimeter microplate reader (Molecular Devices SPECTRA$_{max}$ GEMINI XS) with λ_{ex}=488 nM; λ_{Em}=520 nM and λ_{em}=585 nm. A reduction of at least about 5% in the λ_{em}=585 nm is indicative of BoNT/A protease activity. An increase of about 5% in the λ_{em}=520 nm also is indicative of BoNT/A protease activity of the dichain or light chain botulinum toxin.

Kinetic assays are performed as follows. Several reactions containing the same amount of LC/A or dichain toxin are initiated in the buffer and under the conditions described above. Different reactions are then stopped at two or five minute intervals, and fluorescence detected as described above.

These results demonstrate that botulinum toxin proteolytic activity can be assayed with an intramolecularly quenched FRET substrate.

All journal article, reference and patent citations provided above, in parentheses or otherwise, whether previously stated or not, are incorporated herein by reference in their entirety.

Although the invention has been described with reference to the examples provided above, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the claims.

SEQUENCE LISTING

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<400> SEQUENCE: 1

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1 5

<210> SEQ ID NO 2
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<212> TYPE: PRT
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<400> SEQUENCE: 2

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1 5 10 15

Arg Ala Asp Gln Leu Ala Asp Glu Ser Leu Glu Ser Thr Arg Arg Met
20 25 30

Leu Gln Leu Val Glu Glu Ser Lys Asp Ala Gly Ile Arg Thr Leu Val
35 40 45

Met Leu Asp Glu Gln Gly Glu Gln Leu Glu Arg Ile Glu Glu Gly Met
50 55 60

Asp Gln Ile Asn Lys Asp Met Lys Glu Ala Glu Lys Asn Leu Thr Asp
65 70 75 80

maximum to near said donor fluorophore emission maximum, said shift in emission maxima being indicative of BoNT/A protease activity.

39. The method of claim **29**, wherein said acceptor is a fluorophore and step (c) comprises detecting the ratio of fluorescence amplitudes near an acceptor emission maximum over the fluorescence amplitudes near a donor fluorophore emission maximum, wherein an increase in substrate cleavage results in a decreased ratio of said treated substrate as compared to said control substrate, said decreased ratio being indicative of BoNT/A protease activity.

40. The method of claim **29**, wherein said acceptor is a fluorophore and step (c) comprises detecting the excited state lifetime of the donor fluorophore of said treated substrate, wherein an increase in substrate cleavage results in an increase in donor fluorophore excited state lifetime of said treated substrate as compared to said control substrate, said decreased ratio being indicative of BoNT/A protease activity.

41. The method of claim **29**, further comprising repeating step (c) at one or more later time intervals.

42. The method of claim **29**, wherein at least 90% of said BoNT/A substrate is cleaved.

43. The method of claim **29**, wherein at most 25% of said BoNT/A substrate is cleaved.

44. The method of claim **43**, wherein at most 15% of said BoNT/A substrate is cleaved.

45. The method of claim **44**, wherein at most 5% of said BoNT/A substrate is cleaved.

46. The method of claim **29**, wherein the conditions suitable for clostridial toxin protease activity are selected such that the assay is linear.

47. The method of claim **29**, wherein said donor fluorophore is not positioned within said BoNT/A P_5-P_4-P_3-P_2-P_1-P_1'-P_2'-P_3'-P_4'-P_5' cleavage site sequence.

48. The method of claim **29**, wherein said acceptor is not positioned with in said BoNT/A P_5-P_4-P_3-P_2-P_1-P_1'-P_2'-P_3'-P_4'-P_5' cleavage site sequence.

49. The method of claim **29**, wherein said acceptor is a fluorophore and step (c) comprises detecting the ratio of fluorescence amplitudes near an donor emission maximum over the fluorescence amplitudes near a acceptor fluorophore emission maximum, wherein an increase in substrate cleavage results in an increased ratio in said treated substrate as compared to the control substrate, said increased ratio being indicative of BoNT/A protease activity.

50. The method of claim **29**, wherein said acceptor is a quencher and step (c) comprises detecting donor fluorescence intensity of said contacted cell, wherein an increase in substrate cleavage results in an increase in donor fluorescence intensity of said treated substrate as compared to said control substrate, said increased donor fluorescence intensity being indicative of BoNT/A protease activity.

51. The method of claim **29**, wherein said BoNT/A substrate comprises at most 20 residues.

52. The method of claim **29**, wherein said BoNT/A substrate comprises at most 40 residues.

53. The method of claim **29**, wherein said BoNT/A substrate comprises at most 50 residues.

54. The method of claim **29**, wherein said BoNT/A substrate comprises at most 100 residues.

55. The method of claim **29**, wherein said donor fluorophore and said acceptor are separated by at most 10 residues.

56. The method of claim **29**, wherein said donor fluorophore and said acceptor are separated by at most 20 residues.

57. The method of claim **29**, wherein said donor fluorophore and said acceptor are separated by at most 30 residues.

58. A method of determining protease activity of botulinum toxin serotype A (BoNT/A), comprising the steps of:

(a) treating a BoNT/A substrate with a sample under conditions suitable for clostridial toxin protease activity, said BoNT/A substrate comprising

(i) a donor fluorophore;

(ii) an acceptor having an absorbance spectrum overlapping the emission spectrum of said donor fluorophore; and

(iii) a BoNT/A recognition sequence comprising a BoNT/A P_5-P_4-P_3-P_2-P_1-P_1'-P_2'-P_3'-P_4'-P_5' cleavage site sequence, said BoNT/A P_5-P_4-P_3-P_2-P_1-P_1'-P_2'-P_3'-P_4'-P_5' cleavage site sequence intervening between said donor fluorophore and said acceptor;

wherein either of said donor fluorophore, said acceptor, or both said donor fluorophore and said acceptor are genetically encoded; and

wherein, under the appropriate conditions, resonance energy transfer is exhibited between said donor fluorophore;

(b) exciting said donor fluorophore; and

(c) determining resonance energy transfer of said treated substrate relative to a control substrate, wherein a difference in resonance energy transfer of said treated substrate as compared to said control substrate is indicative of BoNT/A protease activity.

59. The method of claim **58**, wherein said donor fluorophore is genetically encoded.

60. The method of claim **58**, wherein said acceptor is genetically encoded.

61. The method of claim **58**, wherein said donor fluorophore and said acceptor are genetically encoded.

62. The method of either claim **59** or **61**, wherein said donor fluorophore is selected from the group consisting of blue fluorescent protein, cyan fluorescent protein, green fluorescent protein, yellow fluorescent protein and red fluorescent protein.

63. The method of either claim **60** or **61**, wherein said acceptor is a fluorophore.

64. The method of claim **63**, wherein said acceptor fluorophore is selected from the group consisting of blue fluorescent protein, cyan fluorescent protein, green fluorescent protein, yellow fluorescent protein and red fluorescent protein.

65. The method of claim **58**, wherein said BoNT/A P_5-P_4-P_3-P_2-P_1-P_1'-P_2'-P_3'-P_4'-P_5' cleavage site sequence comprises SEQ ID NO: 1.

66. The method of claim **58** or **65**, wherein said sample is isolated clostridial toxin.

67. The method of claim **58** or **65**, wherein said sample is isolated clostridial toxin light chain.

68. The method of claim **58**, wherein said BoNT/A recognition sequence comprises the amino acid sequence selected from the group consisting of SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, amino acid residues 137 to 206 of SEQ ID NO: 2, amino acid residues 134 to 206 of SEQ ID NO: 2 and SEQ ID NO: 2.

69. The method of claim **58**, wherein said sample is a crude cell lysate.

70. The method of claim **58**, wherein said sample is a formulated clostridial toxin product.

71. The method of claim **58**, wherein said sample is formulated BoNT/A product containing human serum albumin.

72. The method of claim **58**, wherein said acceptor is a fluorophore and step (c) comprises detecting donor fluores-